0076019

United States Department of the Interior

FISH AND WILDLIFE SERVICE
Upper Columbia River Basin Field Office
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October 31, 2000

Jamie Zeisloft
U.S. Dept. of Energy
Richland Operations Office
3350 George Washington Wy, HO-12
Richland, WA 99352

Subject: Distribution of Final Report
DOE IA No. DE-AI06-97RL13570

Dear Mr. Zeisloft:

Enclosed please find three (3) copies of the Final Report, "The Potential for Chromium to Adversely Affect Chinook Salmon (*Oncorhynchus tshawytscha*) in the Hanford Reach of the Columbia River, Washington, USA." This report is provided for your distribution. The report was prepared as required under DOE IA No. DE-AI06-97RL13570.

The report contains information on the toxicity of chromium to chinook salmon at three life stages: fertilization, early-life-stage, and parr health. This report is the culmination of nearly three years of planning, implementing, and conducting the studies, and was greatly enhanced by the extensive input from the Hanford Natural Resource Trustee Council.

Please contact Brad Frazier of my staff (509-893-8003) if you have any questions regarding the Final Report.

Sincerely,

Susan B. Martin
Supervisor

USGS
science for a changing world

Columbia Environmental Research Center

THE POTENTIAL FOR CHROMIUM TO ADVERSELY AFFECT CHINOOK SALMON (*Oncorhynchus tshawytscha*) IN THE HANFORD REACH OF THE COLUMBIA RIVER, WASHINGTON, USA.

Prepared for the United States Fish and Wildlife Service
Upper Columbia River Basin Field Office

U.S. Department of the Interior
U.S. Geological Survey

UNITED STATES GEOLOGICAL SURVEY FINAL REPORT:

THE POTENTIAL FOR CHROMIUM TO ADVERSELY AFFECT CHINOOK SALMON (*Oncorhynchus tshawytscha*) IN THE HANFORD REACH OF THE COLUMBIA RIVER, WASHINGTON, USA.

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DISCLAIMER

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SUMMARY

The Hanford Nuclear Reservation in south central Washington was claimed by the federal government as a site for the production of plutonium. During the course of production and operation of the facilities at Hanford, radionuclides and chromium were discharged directly to the river and also contaminated the groundwater. Discharge and seepage of chromium-contaminated groundwater from the 100 Area into the Columbia River may have adverse impacts to salmon and other aquatic life, and remains a concern to area Natural Resource Trustees.

The Hanford Natural Resource Trustee Council in conjunction with the U.S. Fish and Wildlife Service and the U.S. Geological Survey designed this study to assess the effects of chromium (Cr) on chinook salmon (*Oncorhynchus tshawytscha*) under exposure conditions similar to those of the Hanford Reach of the Columbia River. To achieve this objective three studies were conducted with the different life-stages of salmon present in the Hanford Reach. These studies examined chromium effects on 1) Fertilization: Determine the potential for chromium to adversely affect chinook salmon gametes and their fertilization; 2) Early Life-Stages: Determine the effects of chromium on the early development of chinook salmon; and 3) Fish Health: Determine the degree of fish health impairment of chinook salmon parr exposed to chromium. These studies exposed chinook salmon to aqueous chromium concentrations ranging from 0 to 266 µg/L. The current ambient water quality criteria established for the protection of aquatic life (USEPA 1986) is 11 µg/L. Hexavalent chromium concentrations ranging from non-detectable to 632 µg/L have been measured in porewater samples collected from the 100 Areas (Hope and Peterson 1996).

Under exposure conditions designed to closely mimic events that occur in the river, the fertilization of chinook salmon eggs was not affected by concentrations of chromium that ranged from 11 to 266 µg/L. Data suggest that the instantaneous nature of fertilization likely limits the potential effects of chromium on fertilization success.

Early life-stage exposures began with eyed-eggs and continued for 83 days through hatch until the median swim-up date; the approximate life-stage at which salmon emerge from the redd. Fish were then held for 30 days in chromium-free conditions to monitor development, physiological function, growth, and survival. Exposure to aqueous chromium concentrations ranging from 5 to 120 µg/L did not significantly reduce the survival or growth of early life-stage chinook salmon. Behavior, physiological function and development were not significantly impaired. This data is similar to early studies conducted at Hanford that showed alevins to be tolerant to chromium exposure until after the initiation of exogenous feeding and swim-up, when mortality increased dramatically. Based on the results of these studies and the studies conducted earlier at Hanford, it is evident that the chinook salmon are more susceptible to adverse impacts due to chromium exposure during the transition to exogenous feeding and the following period of rapid growth. Our data and previous studies

suggest that the early period of exogenous feeding is among the more sensitive periods to chromium toxicity.

Chinook salmon parr were exposed for 105 days to 24 and 54 µg Cr/L. Neither growth nor survival of parr was affected as a result of exposure to these concentrations for 105 days. On Day 105 concentrations were increased from 24 to 120 µg Cr/L and from 54 to 266 µg Cr/L until the end of the experiment on Day 134. Exposure concentrations were increased after the initial 105 days in an attempt to produce an observable response (e.g., behavioral abnormality, reduced growth, mortality) that could be related to fish health assessment parameters. Weights were affected when parr were exposed to 24/120 µg Cr/L and survival of parr was decreased in the 54/266 µg Cr/L treatment. The health of fish was significantly impaired in both the 24/120 and 54/266 µg Cr/L treatments. Changes were noted in DNA, histology, lipid peroxidation, and necropsies of fish. It appears that chromium accumulates and enters the lipid peroxidation pathway where fatty acid damage and DNA lesions occur to cause cell death and tissue damage. These changes translate into the histopathology and gross changes observed during necropsies. It is apparent that the kidney is the target organ during chromium exposures through the water column. Gross abnormalities were noted in the kidneys, histological lesions in the kidneys were more frequent, products of lipid peroxidation were elevated, and these changes were associated with elevated doses of chromium in the kidney and reduced growth and survival. Additionally, variations in DNA were noted in the blood and are associated with pathological changes in the kidney and spleen. While most of the physiological malfunctions were noted following parr exposures to concentrations $\geq$ 120 µg Cr/L, variations in DNA were observed following exposures to 24 µg Cr/L. The significance of these malfunctions is particularly important because they are associated with impaired growth and reduced survival, which are effects that can be measured at the population level. Therefore, these changes can be used to investigate the health of resident fish in the Hanford Reach of the Columbia River.

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INTRODUCTION

The Hanford Nuclear Reservation in south central Washington is a 900 square km area claimed by the federal government in 1943 as a site for the production of plutonium (Figure 1)(Geist 1995). The location was ideal because it was remote, sparsely populated, and most importantly, had a readily available supply of cold water from the Columbia River. Because of national security concerns, public access and river development projects were restricted until 1971 (Dauble and Watson, 1997). Extensive dam building and development occurred throughout the Columbia River Basin from 1943 to 1971 and led to severely reduced populations of chinook salmon (*Oncorhynchus tshawytscha*). The 90 km section within the Hanford Reservation was not developed, and today, the Hanford Reach remains a free flowing stretch of the Columbia River and is the only remaining area where significant mainstem salmon spawning occurs in the Columbia River (Dauble and Watson 1990). Though upstream dams regulate flows within the Hanford Reach, it is the last unimpounded stretch of the mainstem Columbia River. As a result the use of the Hanford Reach for fall chinook salmon spawning and rearing has dramatically increased since 1960 (Becker 1985, Dauble and Watson 1990). The 10-year average adult escapement increased from 27,660 (1964-1973) to 54,661 (1983-1992). This increase is pronounced when compared with the rest of the mid and upper Columbia River where chinook salmon runs have declined during the same time period.

During operation of the Hanford facilities large quantities of Columbia River water were used to cool nuclear reactors, and cooling water was treated with sodium dichromate to prevent corrosion and mineral collection within the pipes (Peterson et al. 1996). During operations, cooling water with associated radionuclides and chromium were discharged directly to the river and also entered groundwater through leakage of pipes and seepage from retention areas. Today, groundwater at the Hanford site continues to be contaminated with chemical and radiological constituents (Geist et al. 1994). The hydraulic head of the groundwater aquifers in the 100 Area (National Priority List Site) are higher in elevation than that of the Columbia River, which results in discharge from the aquifer into the Columbia River through shoreline springs and seeps (Figure 2). The groundwater is hydraulically connected to the river with peak aquifer discharges occurring during low river flows (fall and winter) and minimum aquifer discharges occurring during high river flows (spring and summer) (Geist et al. 1994). Spawning occurs in close proximity to the 100 Area where contaminated groundwater is entering the river. Redd counts conducted over the last several decades indicate that the majority of chinook salmon spawning occurs over only a few kilometers of river in the Hanford Reach (Dauble and Watson 1997). This intensive habitat use in proximity to contaminated plumes of groundwater strongly suggests that chinook salmon are at risk of exposure. Trustees responsible for fish populations in this stretch of the

river need to be able to adequately assess the potential for contaminated groundwater to impact chinook salmon.

Adult chinook salmon spawn in variable water depths, water velocities, and substrate types (Swan et al. 1988). Spawning in the Hanford Reach begins in mid-October, peaks in mid-November, and ends in late November (Dauble and Watson 1997). Egg and fry development within the redds takes place from mid-October to May during low river flows that result in peak aquifer discharges. Based on the mid-November peak redd abundance and ambient temperatures, eggs would become eyed in early December, hatch in late December, and alevins would emerge from the redds in late February. Upon emergence, fry move out of the main river channel into shallow, slow moving, near shore and backwater habitat (Dauble and Watson 1990, Dauble et al. 1989). Juveniles remain in the Hanford Reach from February to mid-July feeding on macroinvertebrates (Becker 1973). Outmigration begins in May and is usually completed by July at 5-7 months of age, 60-70mm in length, and 3-4g in weight (Olson and Foster 1956).

Chromium is a contaminant of major concern associated with the 100 Area groundwater and seeps. The concentrations of chromium in the groundwater upwellings (Hope and Peterson 1996) exceed the chronic ambient water quality criteria (AWQC, 11 µg/L) for the protection of aquatic life, established by the U.S. Environmental Protection Agency (USEPA 1986). Hexavalent chromium concentrations ranging from non-detectable to 632 µg/L have been measured in porewater samples collected from the 100 Areas (Hope and Peterson 1996). The Department of Energy currently has activities underway to pump and treat chromium at the Hanford facility, and reduce the amounts of hexavalent chromium released into the Hanford Reach. However, the critical nature of the Hanford Reach as spawning habitat for the chinook salmon, makes it essential to determine the potential for chromium in the groundwater to adversely impact chinook salmon (Geist 1997). While some data do exist on the effects of hexavelent chromium on salmon (Olson and Foster 1956, Buhl and Hamilton 1991), previous studies did not investigate the direct effects on fertilization, effects on alevin exposure only, recovery of exposed alevins, or physiological impairment.

The early life stages of chinook salmon are most likely to come in constant contact with elevated chromium and these stages have been shown to be the most sensitive to contaminants (McKim 1977). Chromium may hamper fertilization success by directly acting on the fertilized egg to cause death of the embryo (Billard and Roubaud 1985). Or chromium may react with the sperm and egg individually to impede fertilization. If fertilization is successful, chromium may affect the survival of early lifestages (Olson and Foster 1956, Benoit 1976). While it has been documented that elevated concentrations of chromium reduce survival (Buhl and Hamilton 1991), and growth (Olson 1956, Benoit 1976), information has not been gathered on the relevance of recovery periods on these toxicological effects. In the Hanford Reach, chromium that moves from the groundwater upwellings becomes diluted extensively. Thus, as young fry begin to emerge from the redds,

they may no longer be exposed to elevated concentrations of chromium. The effects of chromium exposure to alevins, as monitored by post-exposure recovery of fry during later development, can mimic the exposure situation present in the Hanford Reach.

Chinook salmon will be present in the Hanford Reach for 5-7 months, and it is important to understand health effects as related to chromium exposure. It is unclear what the exposure concentration might be through contaminated surface water or diet, but long-term health effects from continuous exposure is not well understood in either early life or parr stages (Geist et al. 1994). An understanding of the physiological responses (pathology) associated with chromium exposure can be used to supplement fish population or water and sediment monitoring. Evaluations based on the residue concentrations and physiological condition (e.g. increased lipid peroxidation) of fish integrates the actual exposure to pollutants (dose) and effects of these exposures on fish survival and growth (Farag et al. 1994, Farag et al. 1995). Further associations of tissue chromium accumulation, oxidative stress, and growth reduction would add more weight to a determination of fish health impairment. This weight of evidence approach uses all of the information gathered to determine the health status of a fish population. These fish health assessment tools, developed for in the laboratory under controlled conditions, can then be applied in the field to evaluate the health status of resident fish in the Hanford Reach.

The Hanford Natural Resource Trustee Council in conjunction with the U.S. Fish and Wildlife Service and the U.S. Geological Survey designed this study to assess the effects of chromium on chinook salmon under exposure conditions similar to those of the Hanford Reach of the Columbia River. To achieve this objective we completed three tasks:

Task 1. Fertilization: Determine the potential for chromium to adversely affect chinook salmon gametes and their fertilization.

Task 2. Early Life Stage: Determine the effects of chromium on the early development of chinook salmon.

Task 3. Fish Health: Determine the degree of fish health impairment of chinook salmon exposed to chromium.

METHODS

EXPOSURE CONDITIONS:

Experimental water simulated that of the Columbia River surface and pore water in the Hanford Reach and conditions known to be associated with the location of spawning redds (Hope and Peterson 1996, Geist 1997). Experimental water was adjusted to a hardness of 80 mg/L as $CaCO_3$; pH, alkalinity, and conductivity were maintained in a range consistent with Columbia River conditions. Experimental water temperature matched seasonal conditions: December through March, 5°C; March through July, 10°C (Wiggins et al. 1997). Geist (1997) documented that the hyporheic zone (where river water and groundwater mix) is generally warmer than the river water. However, data from samples collected between November and March indicate that the temperature of the hyporheic zone minus the river water is only 1°C. Experimental water was prepared by blending laboratory well water with deionized water produced by reverse osmosis. Experimental water produced in this way eliminated the use of surface water and the potential for fish pathogens to be introduced to the experiment and influence test results. Experimental water was produced in 5,600L batches and analyzed to insure quality was within 5% of the experimental design in terms of hardness, alkalinity, conductivity, and pH. Unless otherwise indicated, experimental water was used. Photoperiod was adjusted to simulate time of year of the exposure.

The range of chromium (Cr) concentrations tested in the experimental water ranged from 0 to 266 µg/L. These concentrations were above and below the chronic AWQC for chromium, 11 µg/L (USEPA 1986). This concentration range is also representative of concentrations in pore water sampled from the intergravel substrates in locations where salmon spawn (Giest 1997, Hope and Peterson et al. 1996). Specific concentrations are stated with each task.

SELECTION OF TEST ORGANISMS:

Gametes and eyed embryos of fall-run chinook salmon were obtained from the McNenny State Fish Hatchery, Spearfish, South Dakota. Salmon from the McNenny Hatchery were selected for use in these studies by the Trustees based upon consideration of the following:

1.) Chinook salmon from the Hanford Reach may be exposed to significant concentrations of chromium, and several other contaminants during development and residence in the Reach (Geist et al. 1994). The potential effects of these contaminants on chinook salmon are unknown. A history of pre-exposure to environmental contaminants of test organisms could potentially bias or confound test results (ASTM 2000a). Selection of a fall-run chinook salmon stock from the McNenny Hatchery eliminated the potential confounding effects due to possible pre-exposure.

2.) While evolutionary adaptation has resulted in stocks of salmon that exhibit distinct differences in life history and reproductive site fidelity, these ecological adaptations would not likely result in significant differences in the tolerance or sensitivity to anthropogenic contaminants of relatively recent origin, such as hexavalent chromium (Mayer and Ellersieck 1986). Evidence of adaptation to environmental concentrations of chromium may in, and of itself, constitute a biological effect of exposure and result in altered viability of natural populations.

3.) Data collected from controlled laboratory studies at the Columbia Environmental Research Center and the Jackson Field Research Station will be compared to results of planned onsite studies that will increase the environmental realism of exposure by using site-specific test organisms, Columbia River water, and contaminated Hanford Groundwater.

4.) Adult brood fish from the McNenny Fish Hatchery are examined and tested for disease and parasite infection during spawning, and the eggs certified disease free prior to testing or shipment to Columbia, Missouri and Jackson, Wyoming. The disease free status is essential in assuring that toxicity testing is performed on healthy test organisms, increases reliability of results, and is a recommended standard procedure (ASTM 2000b).

5.) Chinook salmon from the McNenny Hatchery have been used as a source for test organisms in past Natural Resource Damage Assessments (Blackbird Mine Site, Idaho; Marr et al. 1995). Use of a single source for test organisms provides a consistent baseline of data that can be applied by Trustees to other contaminant releases and contaminated sites.

STATISTISTICAL INTERPRETATION OF RESULTS:

Statistical analyses were performed using SAS system software, version 6.11 (SAS Institute Inc., Cary, North Carolina), SYSSTAT (SPSS, Chicago, Illinois), or Toxstat 3.4 (West Inc. and University of Wyoming 1994). Multivariate Analysis of Variance (MANOVA) followed by Tukey means comparisons was performed on all data that meet the assumptions of homogeneity and normality. The dependent variables included percent fertilization, survival, growth, concentrations of products of lipid peroxidation, and concentrations of metals in tissues. The number of replicates for each experiment was four (unless otherwise indicated). If the data did not meet these assumptions and could not be transformed to do so, non-parametric statistical analyses were performed. Statistical significance was assigned at $P \leq 0.05$. Acceptance or rejection of test results was determined

from statistical analyses and peer review of the methods, data, and results. The observations noted during the histological examinations were not subjected to statistical analyses. The data were compiled with summary statistics (means and standard errors) and the prevalent and biologically significant observations were reported and discussed. The data for DNA alterations measured by flow cytometry (see below) were analyzed with a weighted least squares procedure (Johnson and Wichern 1988).

TASK 1: FERTILIZATION:

Effects of chromium on fertilization can be investigated with three experiments 1) toxicity of chromium to the ovum, the ovum survival test; 2) toxicity of chromium to sperm, the sperm survival test; and 3) toxicity of chromium to fertilization, the fertilization test. However, experiments 1 and 2 were designed to investigate the cause of toxicity that might be observed during a fertilization test. We did not document toxicity of chromium during preliminary fertilization experiments. Therefore, the trustees agreed that we focus our efforts on the investigation of toxicity during fertilization tests. Snake River cutthroat trout (*Oncorhynchus clarki* spp.) were used as a surrogate species during additional fertilization experiments.

Gametes were taken from Snake River cutthroat brood stock at the Jackson National Fish Hatchery during May 1999 and from chinook salmon brood stock during October 1999. These are normal times for gametogenesis in spring spawning cutthroat trout and fall spawning adult chinook salmon. The stocks were checked weekly for ovum and sperm formation prior to the experiments. Pooled sources of eggs and sperm were collected from 7 female and 8 male cutthroat trout and from 3 female and 10 male chinook salmon.

A standard 1% NaCl (saline) solution was used to maintain active ova and sperm during the fertilization experiment. Six treatment concentrations of chromium: 0, 11, 24, 54, 120, and 266 µg/L were prepared in 1% NaCl. Each treatment was replicated four times for a total of 24 treatments. Ova and sperm were mixed for 1 min followed by rinsing and water hardening in Hanford experimental water according to standard procedures (Piper et al. 1982). Water hardening lasts for approximately one hour and is the process by which water is absorbed into the eggs and fills the perivitelline space between the shell and yoke. The eggs become turgid during this process and additional water exchange is minimal during further development.

Thirty mls of Ova (300-400) were added to the plastic fertilization container (6" diameter). One ml of sperm, and 30 ml of 1% NaCl containing the appropriate chromium concentration was then added to the same container. The mixture was swirled gently for 1 minute. The eggs were rinsed 3X with 25 mls of Hanford experimental water with the appropriate concentration of Cr. The eggs were then allowed to harden in Hanford experimental water with Cr for 1 ½ hr. After water hardening, the eggs were rinsed 3 X with 80 mls of Hanford experimental water lacking chromium, and transferred into incubators.

Eggs from the cutthroat trout were incubated at the USGS Jackson Field Research Station (temperature 10°C; hardness, 158 mg/L as $CaCO_3$; alkalinity, 150 mg/L as $CaCO_3$; and pH, 7.3). Eggs from chinook salmon were incubated in McNenny hatchery water (temperature, 11°C; hardness, 360 mg/L as $CaCO_3$; alkalinity, 210 mg/L as $CaCO_3$; and pH, 7.6). The eggs were cleared in 10% acetic acid solution for at least 2 min and percent fertilization was determined. The embryo of fertilized eggs turns an opaque white and become visible through the translucent chorion. By 10 days the embryos have a definite optic lobe developed with an elongated somite and can be easily distinguished from an unfertilized germinal disk.

The principal investigators decided to use the 1% NaCl solution rather than physiological saline as described in the study protocol. The complex ion mixture affected water chemistry analyses and during 1998 we documented no difference in fertilization success between the 1% NaCl and physiological saline solutions.

TASK 2: EARLY LIFE-STAGE:

Eyed eggs of chinook salmon were exposed to 0, 5, 11, 24, 54, and 120 µg Cr/L. The control treatment was experimental water with no chromium added (0 µg/L). The test was conducted in a modified Mount and Brungs (1967) flow-through diluter system. Temperature was maintained at 5±2 °C by chilling the exposure water before it entered the diluter and submerging the exposure aquaria in a temperature-controlled water bath. Reconstituted exposure water was adjusted to 80 mg /L as $CaCO_3$ hardness to simulate conditions likely to occur in the Hanford Reach of the Columbia River.

To initiate the test (Day 0), two groups of 50 eggs each were placed into 177-mL glass hatching containers and suspended into each of four exposure aquaria (4 replicate groups of 100 eggs each). The aquaria were covered with black plastic to shield the eggs from light during incubation, and gentle aeration was used to provide continuous circulation of the exposure water. On the median hatch date (Day 32), the alevins were released into the exposure aquaria. On the median swim-up date (Day 83), the chromium exposure was discontinued and the alevins were maintained in the aquaria in chromium-free water until 30 days after the median swim-up date (Day 113). Eyed embryos, larvae, and juveniles were handled so as to minimize stress in accordance with the CERC-Columbia Animal Welfare Plan.

During the exposure, egg mortality and hatching were monitored and recorded daily. Dead eggs were removed from the hatching containers and discarded. Alevin mortality and deformities were monitored daily and dead alevins were removed from the aquaria and discarded. The behavior and development of alevins was observed daily to document differences in the sequence and timing of critical developmental stages including; hatch, onset of movement, side plough, upright plough, free swimming, and exogenous feeding following Dill (1977).

Samples of alevins containing 15 fish each were taken from each of the four replicate exposures at the median hatch date (Day 32), during the alevin stage (Day 70), at median swim-up (Day 83) and at the termination of the study (Day 113) for analysis of whole-body chromium residues and lipid peroxidation. Three fish from each replicate were collected at median hatch (Day 83) and at termination (Day 113) for histological analyses. At the termination of the study (30 days post swim-up) all surviving salmon in each treatment were measured for total length and weighed to determine growth. Fish were not fed for 24 h prior to sampling.

TASK 3: FISH HEALTH:

Prior to the experiment, eyed eggs of chinook salmon were maintained in a Heath^{R} incubator at a temperature of 10 ± 2 °C and hardness of approximately 150 mg/L as $CaCO_3$. Mortalities were documented and removed daily. At hatch, the fish were moved to flow-through culture tanks with a flow of 4 L/min. The fish were fed at least a 5% wet weight ration of a commercial biodiet daily. The daily food ration was split between two feedings.

The experimental phase began during the parr stage of fish by randomly distributing 35 fish in each of 12 test chambers receiving experimental water with a flow-through proportional diluter system. The circular chambers had a 20-L capacity with dimensions of 43.2 cm X 35.6 cm and a volume of 20,510 cubic cm. The fish were allowed to acclimate in the experimental chambers for twelve days before the start of the experiment. The experiment was conducted for a period of 134 days beginning with juvenile fish (approximately 60 days post swim-up). Eyed embryos, larvae, and juvenile were handled so as to minimize stress in accordance with the CERC-Columbia Animal Welfare Plan and the Region 6 U.S. Fish and Wildlife Service, Fish Health Policy.

Chromium in stock solutions was delivered to eight test chambers via automatic pipettes (Micromedic Systems AP, Model #25000FW). Two test concentrations of 24 and 54 µg/L chromium (referred to from this point as 24 and 54) were maintained in each of four replicate chambers. Four chambers without chromium added were used for reference. Thus, a total of 12 experimental (four reference, four with 24 µg Cr/L, and four with 54 µg Cr/L) units were maintained until Day 105. Neither growth nor survival of parr was affected as a result of exposure to these concentrations for 105 days. On day 105 concentrations were increased from 24 to 120 µg Cr/L and from 54 to 266 µg Cr/L until the end of the experiment on Day 134. Exposure concentrations were increased after the initial 105 days in an attempt to produce an observable response (e.g., behavioral abnormality, reduced growth, mortality) that could be related to fish health assessment parameters. Each chamber received 8 L/hr for 10 volume additions per day. Experimental units were checked daily for mortality and observations on behavior.

At Day 105 and at the termination of the experiment (Day 134), samples were collected for fish health measurements. An external necropsy assessment was performed on

all sacrificed fish and lengths and weights were recorded. One whole fish was collected from each replicate chamber for measurements of tissue metal accumulation. Samples for histology were collected from 2 fish from each replicated chamber and fixed in 10% neutral buffered formalin. It should be noted that spleen and skin samples were also collected for histological examinations. Gill lamellae, liver (free of the gall bladder), kidney, and intestine were removed immediately from the 10 individual fish. The samples collected for measurements of lipid peroxidation and tissue metals were frozen with liquid nitrogen and stored at -90°C. The sets of 10 samples were ground with liquid nitrogen and composited by tissue to result in one sample from each replicate chamber. Aliquots of these composites were measured for lipid peroxidation and tissue metals.

Blood samples were collected from 10 fish from each replicate for measurements of variations in DNA. Four of these 10 samples were randomly selected and analyzed for variations in DNA. All cells were stained using a modified whole cell method (Clevenger et al. 1985). The frozen red blood cells were thawed rapidly at 37°C and washed twice in phosphate buffer saline. The cells were counted then fixed in 1.0 ml 0.5% paraformaldihyde for 10 minutes at 4°C. The presence of RNA in a sample can interfere with DNA readings. To remove RNA, 0.1 ml of 1.0 mg/ml Rnase was added and the samples were incubated for 20 minutes at 27 °C. The cells were then suspended in propidium iodide, a fluorescent dye that binds to DNA. An Epic Elite Flow Cytometer (Coulter Corp.) with an argon laser (488 nm) was used to quantify DNA. The samples were run at a rate of 150 cells/sec. DNA Check Beads (Coulter Corp.), human lymphocytes and chicken erythrocyte nuclei were used as external biological controls for QA/QC. The data were analyzed with Elite Software (Coulter Corp.) to produce the mean channel of full peak coefficient of variation (CV) values. The CV values represent the variation in approximately 5,000 data points (cells) analyzed for each sample. The difference between the sample CV and the CV of the external or internal control is defined as the CV difference (CV DIF) and was the discriminating statistic (Misra and Easton 1999). Additional samples of fish tissue from the composites described above are currently being analyzed for DNA alterations with gel electrophorhesis. Fish were not fed for 24 h prior to sampling.

ANALYSIS OF WATER AND TISSUE (ALL TASKS):

Treatment water was monitored once per week for dissolved oxygen, pH, alkalinity, hardness, and conductivity. Samples of treatment water were taken weekly to monitor total chromium exposure concentrations. One hundred mL samples of treatment water from each treatment was filtered using a Nalgene® 300 filter holder. Each filtered sample was transferred to a pre-cleaned, 125 ml I-Chem® polyethylene bottle, acidified to 1% HNO_3, and analyzed with ICP-MS. At each time of total chromium sampling, one additional sample was extracted from the low, middle, and high chromium treatments and analyzed for

speciation of chromium. For analysis of chromium in tissue, samples were lyophilized, acid digested with microwave heating, and analyzed by ICP-MS (PE.SCIEX Elan 6000).

Fish tissues were preserved in 10% neutral buffered formalin for histopathological examinations. Each fish was assigned a random number so that tissue processing and the subsequent examinations were performed "blind." Tissues were processed into paraffin, sectioned at 4 μm, stained with hemotoxylin and eosin, and the sections were viewed with light microscopy. For early life-stage fish, major organs retina, brain, gill, gastrointestinal tract, liver, pancreas, skeletal muscle, skin, kidney, heart, and spleen were observed. Lesions were scored as none (0), mild (1), moderate (2), or severe (3) (Appendix 1-2). The scores of 9 – 12 fish were tallied and a mean was computed. Additionally, the epidermal thickness was measured at 400x (to $\pm$2 μm) at six locations on each larva: left and right dorsal, left and right lateral, and left and right ventral. Left and right measurements were combined for overall dorsal, lateral, and ventral means. For parr, kidney, skin, skeletal muscle, gill, liver, intestinal caeca, exocrine pancreas, and spleen were observed. Lesions were scored with the same 4-point scale defined for early life-stage fish.

A fluorometric assay (Dillard and Tappel 1984; Fletcher et al. 1973) was used to measure products of lipid peroxidation. This assay was used previously on fish tissues collected from sites contaminated with metals (Farag et al. 1995) and measures the relative intensity of fluorophores formed during lipid peroxidation. A chloroform-methanol extraction of tissue preceded the fluorometric measurement. Two hundred milligrams of ground tissue were combined with a 2:1 mixture of HPLC-grade chloroform:methanol (7 ml for a 200-mg sample) in a glass homogenizer. The tissue was processed five times in the homogenizer with a glass pestle, diluted with an equal volume of water, and homogenized two additional times. The mixture was then vortexed for 1.5 minutes and transferred to a Corex tube (Note: use of trade names does not imply endorsement by the U.S. Government). The mixture was centrifuged at 1200 X g for 1.5 min and the chloroform layer was removed. Flourescence was measured (Hitachi f-2000) at a wavelength of 435 nm emission during excitation at 340 and 360 nm.

RESULTS

GENERAL:

When added to the water column, chromium did not alter fertilization or alevin development at concentrations equal to the water quality criteria established for the protection of aquatic life (USEPA 1986). Additionally, chromium did not affect salmon fertilization to concentrations of 266 µg/L or alevin growth and survival to concentrations of 120 µg/L. However, weights were affected when parr were exposed to 24/120 µg Cr/L and survival of parr was decreased following a 54/266 µg Cr/L treatment. It is apparent that the kidney is the target organ during chromium exposures through the water column. Gross abnormalities were noted in the kidneys, histological lesions in the kidneys were more frequent, products of lipid peroxidation were elevated, and these changes were associated with elevated concentrations of chromium in the kidney and reduced growth and survival. Additionally, variations in DNA were noted in the blood and are associated with pathological changes in the kidney and spleen. While most of the physiological malfunctions were noted following parr exposures to concentrations $\geq$ 120 µg Cr/L, variations in DNA were observed following exposures to 24 µg Cr/L. A detailed description of the results obtained during each task follows.

TASK 1: FERTILIZATION:

There were no differences in percent fertilization among treatment groups and the reference (Table 1). This observation was similar for both Snake River cutthroat trout and chinook salmon.

Concentrations of total chromium (Cr) and hexavalent chromium (Cr^{+6}) were generally within 10% of the nominal concentrations (Table 2) and duplicate analyses were $\pm$ 20%. The general agreement of Cr^{+6} results with the nominal concentrations confirmed that the majority of Cr remained in the Cr^{+6} form. Percent recoveries of reference solutions and digestion spikes were $\geq$ 97%. Recovery of a Cr^{+6} spike was 100% and Cr^{+3} was virtually 0%, which indicates virtual quantitative elution of Cr^{+6} and sequestration of Cr^{+3} by the AG-50W-X8 cation exchange resin. Quality control was within acceptable limits specified by CERC.

TASK 2: EARLY LIFE-STAGE:

Exposure conditions during this study were within specifications set out in the study protocol. Water temperature in the exposure system ranged between 5.6 and 6.4°C. Alkalinity ranged from 71 to 76 mg/L as $CaCO_3$, conductivity from 187 to 201 µS/cm, hardness from 79 to 82 mg/L as $CaCO_3$, and pH from 7.9 to 8.3. Concentrations of total chromium measured in the exposure chambers (Table 3) agreed well with the nominal concentrations ($\pm$ 10%). Therefore, nominal concentrations will be used from this point.

The results of duplicate samples, dilution checks, reference solutions, analysis spikes, and calibration checks that were measured for QA/QC were acceptable. The general agreement of Cr^{+6} with the nominal concentrations confirmed that virtually all of the chromium remained in the Cr^{+6} throughout the experiment. The results also indicate proper stock chemical make-up and diluter delivery, good mixing characteristics in diluter chambers for each concentrations, and absence of a reduction and/or absorbing environment in diluter receiving aquaria.

Exposure to chromium did not affect hatching success or the time required for exposure groups to reach median hatch. Eyed eggs were exposed to aqueous chromium for 29 to 31 days before reaching median hatch. Mean hatching success was ≥ 93% in all treatments. The number of obviously deformed individuals that survived past hatching was recorded, but did not differ significantly among treatment groups (Table 4). Individuals with deformities were not considered viable alevins and their numbers were subtracted from the total hatching success to arrive at the percentage of viable alevins. Accounting for deformities, the mean percentage of eyed-eggs resulting in viable alevins exceeded 90% in all treatment groups. Survival remained high through swim-up and the subsequent 30-day recovery period (Table 5). Survival was similar among all treatment groups and was ≥ 88% at termination. Treatment groups did not exhibit any measurable differences in behavior or development. There were no differences among groups in the time required to reach median swim-up or initiate feeding. No clinical or diagnostic behavioral abnormalities were observed in exposed or control fish.

Measurements of weight and length were made on salmon sub-sampled for physiological and histological examination at median hatch, during the alevin stage and at median swim-up (Appendix 3-5). This data is limited, but suggests a trend toward reduced weight among chromium-exposed salmon at median hatch and a trend towards reduced length at swim-up. However only reductions in weight at the 24 µg/L treatment (median hatch; Day 32) and in length at the 54 µg/L treatment (swim-up; Day 83) were statistically significant. No differences in length or weight were detected in the limited data set measured from samples collected during the alevin stage (Day 70) . All fish were weighed and measured at the termination of the study (Day 113). There were no differences in either weight or length between control and chromium-exposed salmon at termination (Table 6).

Whole-body concentrations of chromium (Table 7) were similar among all treatment groups sampled at median hatch (Day 32). Chromium in whole-body tissues appeared to increase during the alevin stage (Day 70) and through median swim-up (Day 83). Whole-body chromium appeared to decrease by the termination of the study (Day 113). Whole-body concentrations of chromium were at their highest levels at median swim-up (Day 83), however, there were no detectable differences between concentrations of chromium measured in the control and the highest exposure groups on this date.

Exposure to aqueous chromium did not result in gross lesions or necrosis at the life-stages or concentrations tested in this study. Histological examination of salmon sampled at median swim-up and at the termination of the study revealed only subtle changes primarily related to alterations in development. Epidermal thickness failed to increase in fish exposed to the highest concentration of chromium (120 µg/L) during the recovery period. These same fish exhibited evidence that remodeling necessary during yolk absorption was impaired as shown by lowered mean scores for apoptosis (gene directed cell death) of the mid-ventral skin. As supporting evidence, fish from the highest exposure groups had dermal dysplasia in the mid-ventral skin (i.e., mid-ventral folding of dermal collagen). This change was temporary, because none of the fish sampled at the termination of the study had folded mid-ventral collagen. In addition, gill lamellar epithelium thickness was slightly enhanced in the 120 µg/L treatment at the termination of the study.

There were no significant differences in the relative intensity of products of lipid peroxidation among the treatment groups at median hatch (Day 32), during the alevin stage (Day 70), at median swim-up (Day 83), or at termination of the study (Day 113).

TASK 3: FISH HEALTH:

The general water chemistry was consistent throughout the experiment. Temperature ranged between 9.9 and 11.8°C except for one day where temperature reached 12.8 °C. The range of alkalinity was 76 to 89 mg/L $CaCO_3$, conductivity was 166 to 180 µS/cm, hardness was 76 to 86 mg/L as $CaCO_3$, and pH was 7.6 to 8.0. The concentrations of Cr measured in the chambers throughout the experiment agreed well (± 10%) with the nominal concentrations. Therefore, nominal concentrations will be used from this point. The results of duplicate samples, dilution checks, reference solutions, analysis spikes, and calibration checks that were measured for QA/QC were acceptable. The general agreement of Cr^{+6} with the nominal concentrations confirmed that virtually all of the chromium remained in the Cr^{+6} throughout the experiment. The results also indicate proper stock chemical make-up and diluter delivery, good mixing characteristics in diluter chambers for each concentrations, absence of a reduction and/or absorbing environment in diluter receiving tanks, and proper performance of the ion exchange columns for elution of Cr^{+6} and sequestration of Cr^{+3}.

Survival and growth were not different from the reference at 105 days (Tables 8 and 9). However, survival decreased to 69.8 % in fish exposed to 54/266 for 134 days. There was also a trend of decreased survival in the 24/120 treatment at 134 days (84.3%) compared to the reference (96.8%). By Day 134, the mean weight of fish in the 24/120 treatment was, 6.8 g, significantly less than the reference (9.6 g). However, fish in the 54/266 treatment weighed 8.5 g, and were not significantly different in size than the reference fish. All measurements performed for the 24 and 24/120 µg/L treatments contain 3 rather than 4 replicates. One replicate failed before the Day 105, the first sampling day of the parr experiment. The fish died rapidly between days 101 and 105 and the cause of these

mortalities is unknown. Histological examinations were performed on the few remaining fish but did not provide information about the cause of death. Additionally, the water chemistry did not indicate any dysfunction of the diluter. Therefore, these mortalities were not included in the survival calculations and there were no fish tissues available from the fourth replicate for the physiological measurements.

The concentrations of chromium in the organs of fish increased when fish were exposed to chromium in the water (Table 10). At Day 105, gill, kidney, and whole body tissues from the 54 µg Cr/L treatment had elevated concentrations of chromium (Note: all tissue data in µg Cr/g dry wt.) (25.7, 25.6, and 8.8 respectively) compared to fish in water without chromium (7.4, 5.5, 1.5 respectively). At Day 134 gill, kidney, liver, and pyloric caeca from fish in the chromium treatments were elevated above the reference fish (Table 10). And fish from the 54/266 treatment had greater concentrations of chromium in gill, liver, and pyloric caeca than those from the 24/120 treatment. However, there was no longer a significant increase in whole body concentrations of chromium at Day 134.

We observed gross changes in the kidneys during the necropsies. The most significant finding was discoloration where white markings bordered the kidney along all edges. This abnormality was noted in 2%, 9%, and 19% of fish from the 54, 24/120, and 54/266 µg Cr/L treatments. None of these markings were noted in fish from the reference or the 24 µg Cr/L treatment.

Lesions observed in gill, kidney, and spleen of fish exposed to chromium during this experiment indicate that cell death occurred in these organs (Tables 11 and 12). Interstitial blood-forming cells, also called hematopoietic cells, are normally present in the kidney. Therefore, a score of 1 (mild) represents a normal condition while a score of less than 1 indicates possible anemia or a decreased ability of fish to fight disease. The scores of fish from the reference chambers were at or near 1, but fish from the 24/120 and 54/266 had mean scores of 0.44 and 0.67 respectively. Necrosis (death), fibrosis (scarring), and dilation of the lumen of the tubules were all noted in kidneys from 24/120 and 54/266 but not in the reference. All of these lesions indicate malfunction of kidney cells in fish exposed to 24/120 and 54/266.

The thickness of the gill lining was greater in the 24/120 (0.44) and 54/266 (0.25) exposures compared to the reference (0.08). Also, apoptosis is a genetically programmed cell death and was more prominent in chloride cells of gills from 24 (0.67), 24/120 (0.67) and 54/266 (0.83) compared to the reference (0.17). The greater amounts of congestion noted in spleens from 54/266 (0.42) compared to the reference (0.17) indicate anemia as did the lower scores for interstitial blood-forming cells in the kidneys of fish from this treatment group. Based on the staining pattern of eosinophilic spherules in the spleens of fish from 54/266, the spherules indicate erythrocyte membrane damage and/or decreased erythrocyte life span. These erythrocytes may be apoptotic.

Additional histological changes were noted in the liver and pancreas (Table 13) but these changes were not as extensive as those found in the gill, kidney, and spleen. Changes were noted in the liver and pancreas of fish from 24 and 54 at Day 105. Hepatocyte glycogen stores were depleted and hepatocytes had lipid accumulations. The distribution of lipid in these livers was irregular and the reference fish had no lipid accumulation in their livers. Thus, this lipid accumulation appears to be pathological. Liver changes were not observed in fish from 24/120 or 54/266 at Day 134. However, the glycogen depletion increased in reference fish from Day 105 to Day 134 but remained unchanged in fish from the treatments groups between Day 105 and Day 134. Reference fish may have depleted glycogen stores as a result of the energy demands of smoltification. However, the glycogen stores in fish from the treatment groups remained unchanged.

Zymogen granules store digestive enzymes that are released after feeding. Therefore, slight depletion is expected in normally feeding fish and was observed in fish from the reference (mean score = 1.00 at Day 105). However, fish that are not feeding or digesting consistently might accumulate zymogen granules in the pancreatic cells, resulting in lower scores for zymogen depletion. Lesser scores for zymogen depletion were observed in the 24 (0.67) and 54 (0.58) compared to the reference (1.00) at Day 105. The zymogen depletion scores decreased in the reference between days 105 and 134. However, the presence of these granules remained unchanged for the treatment groups between days 105 and 134. This phenomenon may define delayed smoltification in chinook salmon exposed to chromium.

The CV DIF in DNA measurements decreased with dose at Day 105 (Table 14). The significantly smaller numbers indicate greater amounts of DNA damage in fish exposed to 24 and 54 μg Cr/L when compared to the reference (1.218 and 1.130 vs. 1.730). There was a negative relationship among treatment groups for variations in DNA at Day 134. The CV DIF values actually decreased significantly when compared to the reference. While these results appear counterintuitive, the death of cells with severely damaged DNA would significantly decrease the CV DIF value. Therefore, cell death in the kidney and spleen, noted in the histological examinations, could result in smaller CV DIF values. We also investigated DNA strand breakage with the gel electrophoresis method. No significant differences were noted in gill and liver using this method (Appendix 6).

There were no significant differences in the relative intensity of products of lipid peroxidation among the treatment groups for gill, liver, pyloric caeca, or whole fish. We were able to collect enough kidney tissue to perform one measurement of lipid peroxidation for each treatment. The relative intensities were reference = 76.5, 120 = 134.8, 266 = 137.7 at a wavelength of 340 excitation and reference = 53.9, 120 = 110.7, and 266 = 112.9 at a wavelength of 360 excitation. These measurements indicate an increase in lipid peroxidation in the kidneys but statistical analyses could not be used to define the differences.

DISCUSSION

GENERAL:

We investigated the effects of chromium at three stages of development in salmon: fertilization, early life-stages, and parr. And we studied the target organs and actions of toxicity by chromium. The instantaneous nature of fertilization likely limited the effects of chromium on fertilization success. Also, chinook salmon appear to grow and survive at normal rates when exposed from the alevin to the swim-up stage to 120 µg Cr/L in the water column. Results of the Parr Health Study clearly indicate that the kidney is an important target organ during aqueous chromium exposures. Changes in the kidney during parr exposure to chromium suggest that variations in DNA, lipid peroxidation, and tissue pathology work to cause effects on growth and eventually death of fish. These changes can be used to investigate the health of resident fish in the Hanford Reach of the Columbia River. Further discussions of each task follow.

TASK 1: FERTILIZATION:

Fertilization of chinook salmon and Snake River cutthroat trout was not affected by concentrations of chromium that ranged from 11 to 266 µg/L. These results were similar for both species and the experiments were conducted at different times. We found similar results during preliminary experiments (data not presented). Billard and Roubaud (1985) defined that ova from rainbow trout (*Oncorhynchus mykiss*) are less sensitive to chromium exposures than sperm. Additionally, fish sperm begin to die soon after activation and are washed away quickly by river currents. Because of the instantaneous nature of fertilization, the limited contact of sperm with the exposure water likely limited the effects of chromium in the water column on fertilization success.

Our results do not agree with fertilization results obtained by Billard and Roubaud (1985) where they documented that 5 µg Cr/L reduced fertilization percentages in rainbow trout. There were some differences in exposure times and species used during these two studies. The time allowed for exposure to chromium during fertilization was shorter during the current study (1 minute versus 15 minutes during Billard and Roubard 1985). This shorter time more closely mimicked fertilization events that occur in river conditions. The ova were held in exposure water for 1 ½ h following fertilization during the current study. This step was included because eggs continue to absorb water for approximately 1 ½ h following fertilization. The ova were not exposed to chromium during the water hardening in the study performed by Billard and Roubard (1985). Furthermore, cutthroat trout and chinook salmon were studied during the present experiments while rainbow trout were used by Billard and Roubard (1985).

TASK 2 EARLY LIFE-STAGE:

Exposure to aqueous chromium concentrations ranging from 5 to 120 µg Cr/L did not significantly reduce the survival or growth of early life-stage chinook salmon. Eyed eggs and the resulting larvae were continuously exposed to these concentrations through the transition to free-swimming, exogenously feeding juveniles: a total of 83 days. Hatching success (≥ 90%) and long-term survival (≥ 88%) was high in all treatments. Data indicates that chromium exposure may have influenced growth early on in the study, but any effects were transitory and differences in fish length and weight were similar in all groups 30 days following the termination of chromium exposure. This data is in general agreement with earlier studies by Olson and Foster (1956) that exposed chinook salmon and rainbow trout to aqueous chromium concentrations to 180 µg/L. In these studies eyed-eggs were unaffected by chromium exposure. Significant mortality among chinook salmon at the highest concentrations began to occur only after swim-up and the onset of exogenous feeding. This is the very time at which our chromium exposure ended. Olson and Foster (1956) also reported significant reductions in growth after 16 weeks of exposure to chromium as low as 16 µg/L. Obviously contaminant effects on growth rates during the periods of most rapid growth will result in increasing absolute differences in body size over time. Our study was designed based upon the assumption that chinook salmon would emerge from the redd at swim-up and no longer be exposed to chromium in upwelling groundwater. Therefore our study did not expose salmon during this critical phase of rapid growth; therefore the timing of the exposure may have diminished the likelihood of observing significant growth reductions.

Temperature may have also influenced the results. Water temperatures in our study were held constant at 5±2 °C, in part to reflect seasonality and the influence of groundwater flow through interstitial spaces. Comparison of the two studies with rainbow trout and chinook salmon conducted by Olson and Foster (1956) indicates that higher water temperatures (>10°C) may have exacerbated the toxicity of chromium to rainbow trout resulting in mortality earlier and at lower concentrations than observed in chinook salmon exposed at lower temperatures.

These data suggest that the timing of chromium exposure and the environmental conditions prevalent in the habitat occupied by salmon during that period are critical in determining the toxicity of chromium. It is apparent that the early weeks of the swim-up life-stage, when salmon are initiating exogenous feeding, is among the more sensitive periods to chromium toxicity. Application of these data to determine the potential for chromium to adversely affect early life-stage salmon in the field should include the life-stages present, the extent of their proximity to chromium sources, and a realistic approximation of the prevailing environmental conditions within that habitat.

Data from our study indicates that chromium may have accumulated in the tissues of early life-stage salmon at the highest concentrations. However the overall concentrations were low. This may be due in part to tissue-specific accumulation of chromium, and to the small size of the fish tested. Parr health studies conducted at Jackson suggest that chromium does accumulate in specific organs and that whole-body residues may not be an appropriate indicator of chromium toxicity in very early life-stage salmon. Histological examination suggests that some specific developmental changes may have occurred in fish exposed to aqueous chromium. However these effects were subtle and not significant enough to cause observable effects in behavior, growth or mortality.

Lipid peroxidation assays did not provide empirical support for the existence of a physiological malfunction that could be related to tissue chromium, growth or survival. This again may be due to the timing of the exposure, the tissue or organ-specific toxicity of chromium and the small size of the fish tested.

TASK 3 FISH HEALTH:

When chinook salmon are exposed to dissolved hexavalent chromium the metal accumulates in various tissues and ultimately causes variations in DNA, lipid peroxidation, histological lesions, gross abnormalities, reductions in weight and reductions in survival. The physiological changes observed during this experiment are most notable because they are associated with reductions in weights and survival that were also observed during this laboratory study. Therefore, we have provided laboratory support for the link between physiological malfunction manifested at the level of the individual and changes in weight and survival that may be manifested at the population level. This information can be used to link changes in resident fish with possible alterations in populations of fish.

Several types of physiological malfunction were noted during this study. We documented two patterns of DNA damage. First, more variation in the DNA compared to the reference resulted when chinook salmon were exposed to 24 or 54 µg Cr/L. Second, less variation in the DNA compared to the reference resulted when chinook salmon were exposed to 24/120 and 54/266 µg Cr/L. Al-Sabti et al. (1994) observed a marked decrease in micronuclei frequency in Prussian carp exposed to 500 µg Cr/L. One might mistakenly suggest that the reduction in the number of micronuclei (a measurement similar to a lower CV DIF value in the current study) indicates less DNA damage when cells are exposed to high concentrations of chromium. However, chromium appears to increase cytotoxicity by enhancing cell death. Chromium can form intermediates that react with DNA (Outridge and Scheuhammer 1993, Bridgewater et al. 1994, Xu et al. 1996, Singh et al 1998). These intermediates can inhibit DNA replication (Patierno et al. 1993) and the DNA repair mechanism (Bridgewater et al. 1994). It is the inhibition of the DNA repair mechanism that may trigger apoptosis (genetically programmed cell death). Apoptosis generally acts to

remove genetically compromised cells from the population. The enhancement of apoptosis can result in greater cell death when tissues are exposed to chromium.

Although the salmon were 30 days older at the end of the 24/120 and 54/266 µg Cr/L exposures compared to the ages of the salmon at the end of the 24 and 54 µg/Cr exposures, it does not appear that age of fish is responsible for the changed pattern noted for DNA strand breakage. First, this pattern of significantly less variation in DNA strand breakage as a result of chromium exposure is supported by the literature (Al-Sabti et al. 1994) and has also been observed by Countryman and Heddle (1976) as a result of radiation exposure and by Easton et al. (1997) as a result of pulp mill effluent. Second, it is not likely that an enhanced DNA repair mechanism as a result of acclimation explains our findings. If the DNA repair mechanism was enhanced, we might have observed similar variations in DNA among the controls, 24/120 and 54/266. However, we observed a significant decrease in the variation of DNA in fish exposed to 24/120 and 54/266 µg Cr/L when compared to fish from the controls. The fish in the control and exposures were identical in ages. Thus, for an enhanced DNA repair mechanism to explain these results it would be necessary for the DNA repair mechanism to not only be enhanced, but to overshoot the level found in the control fish. This is a theory not supported by the literature or our additional histological data that suggest that cell death is responsible for the decrease in DNA variation observed at the termination of the parr health experiment.

Cell death was a pathology noted during the histological examinations. The greatest extent of histopathology was observed in the kidneys of chinook salmon exposed to 24/120 and 54/266 µg Cr/L. Extensive damage was manifested in the kidneys and expressed as decreases in numbers of blood forming cells, cell death (necrosis), and scarring (fibrosis) around kidney tubules. Histological analyses also revealed eosinophilic spherules in the spleen of chinook salmon exposed to 24/120 and 54/266 µg Cr/L. These spherules indicate membrane damage in the erythrocytes that can result from apoptosis of those cells. The histological analyses defined the kidney as the primary site of pathology in fish held in the 24/120 and 54/266 µg Cr/L treatments. This finding is supported by the necropsy results where gross abnormalities were noted in the kidneys (9% and 19% in 24/120 and 54/266 µg Cr/L respectively). The histological analyses also support our finding that cell death is responsible for the noted pattern of DNA damage in this study.

Lipid peroxidation can lead to cell death and tissue damage (Halliwell and Gutteridge 1985, Wills 1985). We noted elevated lipid peroxidation in the kidneys of fish following exposures to 24/120 and 54/266. We did not collect enough kidney tissue to define these differences statistically or to measure this response in the 24 and 54 µg Cr/L treatments. However, the elevated lipid peroxidation response in the kidney that we observed provides supporting evidence that Cr accumulated in the kidney causes pathology through the peroxidation pathway. Lloyd et al. (1997, 1998) have strongly implicated the Fenton reaction in the mechanism for DNA damage during chromium exposures. The Fenton

reaction is a step in lipid peroxidation where transition metals, such as Cr, reduce hydrogen peroxide to hydroxyl radicals. These hydroxyl free radicals generate oxidative DNA lesions and cause the peroxidation of polyunsaturated fatty acids located in cells membranes. The damage to cell membranes can decrease fluidity, increase leakiness, and inactivate membrane-bound enzymes. An ultimate result may be cell death and tissue damage (Halliwell and Gutteridge 1985, Wills 1985). Other researchers have documented that chromium can cause lipid peroxidation (Susa et al. 1996). And we have now documented DNA damage, lipid peroxidation, and necrosis of kidney cells simultaneously as a result of chromium exposure.

Changes in the population level effects, weights and survival, were not observed in fish from 24 and 54 µg Cr/L at Day 105, instead, more subtle changes in DNA, histology, and necropsies were observed. The variation in DNA was elevated and indicates DNA damage (Countryman and Heddle 1976 and Easton et al. 1997). The amount of lipid increased in the livers of the exposed fish at Day 105 and zymogen granule depletion was less in the 24 and 54 µg Cr/L treatments compared to the reference fish at Day 105. This reduction in zymogen granule depletion indicates that fish in these treatments are not digesting food as consistently as the reference fish. These results together with the small number of abnormal necropsies of kidneys from the 54 µg Cr/L treatment suggest that the health in fish from these treatments is slightly impaired compared to the reference fish.

Some of the histological changes also suggest that smoltification may progress at different rates in fish treated with chromium compared to the reference. Glycogen depletion increased between days 105 and 134 in the reference fish, a finding that is consistent with the energy demands of smoltification. However, glycogen depletion remained unchanged at Day 134 in fish exposed to chromium. This same pattern was noted in the pancreas where zymogen granule depletion in 24 and 54 µg Cr/L treatments was different from the reference fish at Day 105. Granule depletion remained unchanged between days 105 and 134 in the exposed fish. In this case a decrease in the depletion of zymogen granules, as noted in the reference fish between days 105 and 134, may indicate a normal progression of smoltification.

In summary, the health of fish was significantly impaired in the 24/120 and 54/266 µg Cr/L treatments. Changes were noted in DNA, histology, lipid peroxidation, and necropsies of fish. It appears that chromium accumulates and enters the lipid peroxidation pathway where fatty acid damage and DNA lesions occur to cause cell death and tissue damage. These changes translate into histopathology and gross changes observed during necropsies. The significance of these malfunctions is particularly important because they are associated in this study with changes in growth and survival, which can be related to effects at the population level. Therefore, these parameters may be used in the field to evaluate the health status of resident fish in the Hanford Reach of the Columbia River.

ACKNOWLEDGEMENTS

Micheal Easton, Ecogen Inc., measured DNA strand breakage with flow cytometry and Gary Marty, UC Davis, provided the histological examinations. Thomas May measured the chromium in tissues and water. Brad Mueller and Richard Skinker provided excellent technical assistance throughout all three tasks.

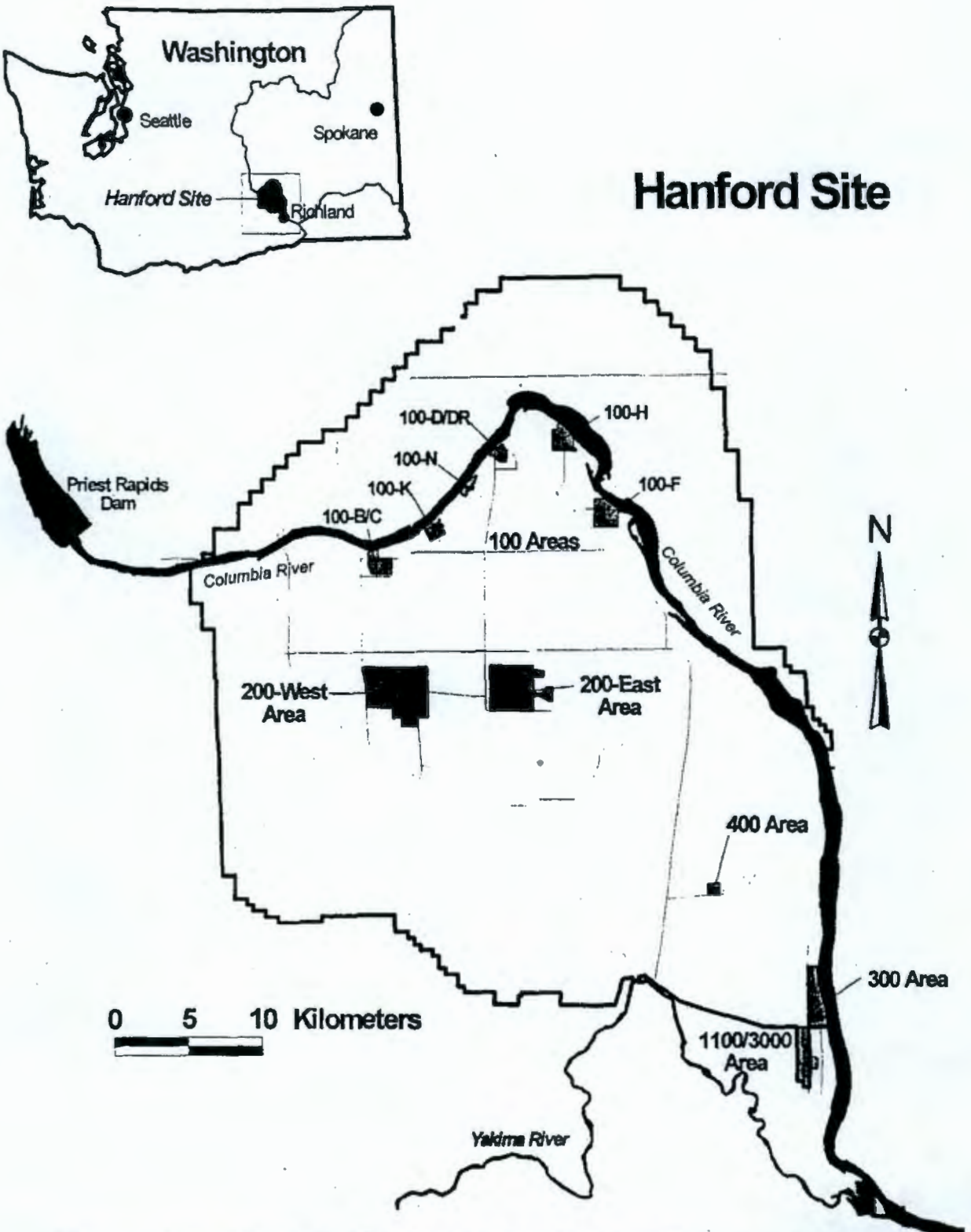

Figure 1. Map of the Hanford Reach of the Columbia River, Washington, USA. Source: Hanford Geographic Information System, Environmental Technologies Data Management, Bechtel Hanford, Inc.

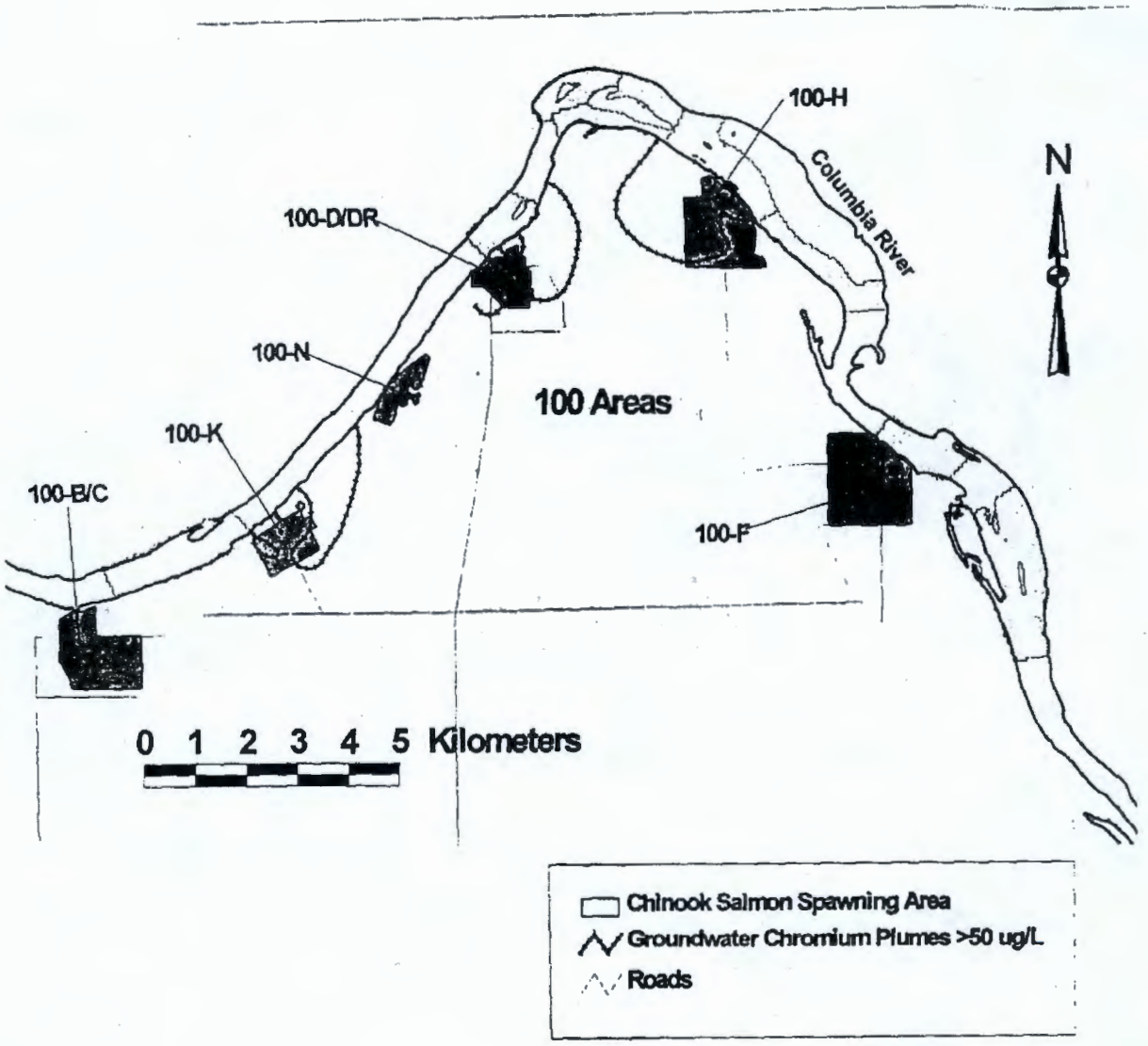

Figure 2. Map of the Hanford Reach of the Columbia River, Washington, USA that flows through the 100 Areas. Crosshatched areas within the river indicate locations of chinook salmon spawning redds. Groundwater plumes with levels of chromium exceeding 50 µg/L are indicated by hatched contour lines. Source: Hanford Geographic Information System, Environmental Technologies Data Management, Bechtel Hanford, Inc.

Table 1. Mean percent fertilization of Snake River cutthroat trout and chinook salmon. The eggs and sperm were exposed to various concentrations of hexavalent chromium during fertilization and the eggs were hardened in water that contained similar concentrations of chromium. Standard error of the mean are in parentheses and different letters denote significant difference at $P \leq 0.05$. Species were analyzed for significance separately. NS = no sample. NOTE: Letter designations are used in this table and those that follow to define the multiple comparisons that were performed with the Tukey analyses. Therefore, no statistical differences were observed when all means are followed by an "a."

Snake River Cutthroat			Chinook Salmon		
Chromium (µg/L)	N	Percent Fertilization	Chromium (µg/L)	N	Percent Fertilization
0	8	47.9[a] (5.7)	0	4	66.7[a] (2.3)
11	4	54.9[a] (4.2)	11	4	67.2[a] (3.1)
24	4	50.3[a] (7.4)	24	NS	NS
54	4	51.4[a] (5.7)	54	NS	NS
120	4	39.0[a] (5.9)	120	4	62.6[a] (2.8)
266	4	51.6[a] (2.7)	266	4	69.1[a] (1.1)

Table 2. Concentrations of total Cr and Cr^{+6} in the water used to expose eggs and sperm to hexavalent chromium during fertilization.

Snake River Cutthroat			Chinook Salmon		
Nominal Chromium (µg/L)	Chromium 1% NaCl Solution	Chromium in exposure water	Nominal Chromium (µg/L)	Chromium 1% NaCl Solution	Chromium in exposure water
0	1.1	<0.5	0	1.6	3.0
11	11.5	10.2	11	12.9	13.9
24	21.4	23.4	24	25.7	26.8
54	55.2	51.2	54	53.0	55.4
120	126.0	117.0	120	113.0	117.0
266	259.0	279.0	266	261.0	251.0
120 Cr^{+6}	135	130			

Table 3. Concentrations of total Cr and Cr^{+6} in the water used to expose early life-stages to chromium. NS indicates that Cr^{+6} concentrations were not sampled for that treatment.

Nominal Chromium (µg/L)	Measured Total Chromium (µg/L)	Measured Cr^{+6} (µg/L)
0	<1.5	NS
5	5.1	7.2
11	11.6	NS
24	25.2	29.8
54	56.5	NS
120	123.2	133.4

Table 4. Mean time to median hatch, percent of eggs hatching, percent of eggs hatching and resulting in viable alevins, and percentage of live-hatched alevins exhibiting obvious deformities (non-viable). Chinook salmon were exposed to 0, 5, 11, 24, 54 and 120 µg Cr/L. Different letter designations indicate a significant difference at $P \leq 0.05$. SEM in parentheses.

Nominal Chromium (µg/L)	Days to Median Hatch	N	Percent Hatched-Live	Percent Viable Hatch	Percent Deformity
0	30.0	4	95.2[a] (1.2)	93.8[a] (1.5)	1.5[a] (0.3)
5	30.8	4	95.5[a] (1.6)	92.2[a] (1.5)	3.25[a] (0.6)
11	31.0	4	93.0[a] (1.4)	90.8[a] (1.1)	2.25[a] (0.5)
24	30.0	4	94.2[a] (0.5)	92.0[a] (0.7)	2.25[a] (0.5)
54	30.5	4	94.5[a] (1.0)	91.2[a] (1.4)	3.25[a] (1.1)
120	30.5	4	93.8[a] (2.3)	92.0[a] (2.4)	1.75[a] (0.5)

Table 5. Mean time to swim-up, cumulative percent of salmon alevins surviving to swim-up, and percent surviving through 30 days post swim-up. Chinook salmon were exposed to 0, 5, 11, 24, 54 and 120 µg Cr/L. Different letter designations indicate a significant difference at $P \leq 0.05$. SEM in parentheses.

Nominal Chromium (µg/L)	Days to Median Swim-up	N	Percent Survival to Swim-up	Percent Survival to 30 Days Post Swim-up
0	83.0	4	92.9[a] (1.5)	92.9[a] (1.5)
5	82.5	4	91.2[a] (1.8)	90.6[a] (2.3)
11	82.5	4	90.1[a] (1.5)	90.1[a] (1.5)
24	83.0	4	88.8[a] (2.0)	88.2[a] (2.4)
54	82.8	4	90.4[a] (1.7)	90.4[a] (1.7)
120	83.5	4	91.0[a] (3.0)	91.0[a] (3.0)

Table 6. Mean weights and lengths of chinook salmon 30 days following the median swim-up date and the termination of chromium exposure. Chinook salmon were exposed to 0, 5, 11, 24, 54 and 120 µg Cr/L. Different letter designations indicate a significant difference at $P \leq 0.05$. SEM in parentheses.

Nominal Chromium (µg/L)	Days	N	Weight (g)	Length (mm)
0	113	4	0.342[a] (0.007)	37.3[a] (0.2)
5	113	4	0.341[a] (0.007)	37.4[a] (0.2)
11	113	4	0.333[a] (0.007)	37.2[a] (0.2)
24	113	4	0.332[a] (0.007)	37.4[a] (0.2)
54	113	4	0.335[a] (0.007)	37.2[a] (0.2)
120	113	4	0.331[a] (0.007)	36.9[a] (0.2)

Table 7. Mean whole-body concentrations of chromium (µg/g) in chinook salmon sampled at median hatch (Day 32), during the alevin stage (Day 70), at median swim-up (Day 83) and at termination of the study (Day 113). Chinook salmon were exposed to 0, 5, 11, 24, 54 and 120 µg Cr/L. Different letter designations indicate a significant difference at $P \leq 0.05$. SEM in parentheses.

Nominal Chromium (µg/L)	N	Median Hatch (Day 32)	Alevin Stage (Day 70)	Median Swim-up (Day 83)	Termination (Day 113)
0	4	0.43[a] (0.09)	0.42[a] (0.11)	0.97[a] (0.28)	0.24[a] (0.03)
5	4	0.30[a] (0.02)	0.36[a] (0.09)	0.35[a] (0.07)	0.22[a] (0.03)
11	4	0.30[a] (0.06)	0.32[a] (0.01)	0.34[a] (0.06)	0.22[a] (0.03)
24	4	0.32[a] (0.04)	0.31[a] (0.02)	0.56[a] (0.05)	0.28[a] (0.02)
54	4	0.62[a] (0.23)	0.45[a] (0.02)	1.14[a] (0.33)	0.37[a] (0.05)
120	4	0.46[a] (0.02)	0.76[b] (0.04)	1.04[a] (0.07)	0.65[b] (0.06)

Table 8. Percent survival of chinook salmon during an experiment where parr were exposed to 24 or 54 µg Cr/L to Day 105. The concentrations of chromium were increased from 24 to 120 µg Cr/L and from 54 to 266 µg Cr/L for the remainder of the experiment that ended on Day 134. Different letter designations indicate a significant difference at $P \leq 0.05$ within a sample day. SEM in parentheses.

Nominal Chromium (µg/L)	Day	N	Percent Survival
0	105	4	87.8[a] (4.0)
24	105	3	92.3[a] (3.7)
54	105	4	91.3[a] (0.5)
0	134	4	96.8[a] (1.9)
24/120	134	3	84.3[a] (5.8)
54/266	134	4	69.8[b] (3.7)

Table 9. Mean weight and lengths of chinook salmon during an experiment where parr were exposed to 24 or 54 µg Cr/L to Day 105. The concentrations of chromium were increased from 24 to 120 µg Cr/L and from 54 to 266 µg Cr/L for the remainder of the experiment that ended on Day 134. Different letter designations indicate a significant difference at $P \leq 0.05$ within a sample day. SEM in parentheses.

Nominal Chromium (µg/L)	Day	N	Weight (g)	Length (mm)
0	105	4	6.0[a] (0.5)	86.3[a] (2.1)
24	105	3	6.0[a] (0.2)	87.3[a] (0.3)
54	105	4	4.7[a] (0.6)	84.3[a] (1.9)
0	134	4	9.6[a] (0.24)	100.8[a] (2.3)
24/120	134	3	6.8[b] (0.6)	93.0[a] (3.2)
54/266	134	4	8.5[a] (3.7)	98.0[a] (2.7)

Table 10. Mean concentrations of chromium in various tissues collected during an experiment where chinook salmon parr were exposed to 24 or 54 µg Cr/L to Day 105. The concentrations of chromium were increased from 24 to 120 µg Cr/L and from 54 to 266 µg Cr/L for the remainder of the experiment that ended on Day 134. Different letter designations indicate a significant difference at $P \leq 0.05$ within a tissue and within a sample day. SEM in parentheses.

Nominal Chromium (µg/L)	Day	N	Gill	Kidney	Liver	Pyloric Caeca	Whole Body
0	105	4	7.4[a] (3.8)	5.5[a] (2.6) N=3	0.9[a] (0.4)	0.6[a] (0.2) N=3	1.5[a] (0.4)
24	105	3	14.8[a] (6.7)	9.6[a] (4.8)	4.0[a] (2.1)	0.7[a] (0.2)	6.6[a] (2.4)
54	105	4	25.7[b] (1.5)	25.6[b] (5.6)	3.0[a] (0.4)	1.2[a] (0.2)	8.8[b] (2.1)
0	134	4	1.3[a] (0.3)	2.8[a] (1.0)	0.4[a] (0.1)	0.3[a] (0.1)	0.2[a] (0.1)
24/120	134	3	29.4[b] (2.0)	30.2[b] (7.9)	4.6[b] (0.6)	2.1[b] (0.4)	6.4[a] (2.7)
54/266	134	4	35.4[c] (1.1)	29.6[b] (2.3)	7.2[c] (0.5)	3.7[c] (0.4)	6.5[a] (3.6)

Table 11. Lesions observed in the kidney of chinook salmon parr that were exposed to 24 or 54 µg Cr/L to Day 105. The concentrations of chromium were increased from 24 to 120 µg Cr/L and from 54 to 266 µg Cr/L for the remainder of the experiment that ended on Day 134. SEM are in parentheses. The means of lesions scored as none (0), mild (1), moderate (2), or severe (3) are presented. Refer to Appendix 1 and 2 for a glossary of terms and guidelines for scoring sections.

Nominal Chromium (µg/L)	Day	N	Kidney			
			Interstitial blood-forming cells	Necrosis (death) of cells lining tubules	Fibrosis (scarring) around tubules	Dilation of tubular lumen
0	105	12	1.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
24	105	9	0.78 (0.15)	0.00 (0.00)	0.00 (0.00)	0.11 (0.11)
54	105	12	0.92 (0.08)	0.00 (0.00)	0.00 (0.00)	0.08 (0.08)
0	134	12	0.92 (0.08)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
24/120	134	9	0.44 (0.18)	0.56 (0.29)	0.44 (0.34)	0.33 (0.24)
54/266	134	12	0.67 (0.14)	0.50 (0.19)	0.42 (0.23)	0.50 (0.19)

Table 12. Lesions observed in the gill and spleen of chinook salmon parr that were exposed to 24 or 54 µg Cr/L to Day 105. The concentrations of chromium were increased from 24 to 120 µg Cr/L and from 54 to 266 µg Cr/L for the remainder of the experiment that ended on Day 134. SEM are in parentheses. The means of lesions scored as none (0), mild (1), moderate (2), or severe (3) are presented. Refer to Appendix 1 and 2 for a glossary of terms and guidelines for scoring sections.

Nominal Chromium (µg/L)	N	Day	Gill		Spleen	
			Lamellar hypertrophy	Apoptosis (death) of chloride cells	Congestion	Eosinophilic spherules; apoptotic erythrocytes
0	12	105	0.17 (0.11)	0.33 (0.14)	1.00 (0.17)	0.08 (0.83)
24	9	105	0.56 (0.17)	0.67 (0.17)	1.11 (0.11)	0.00 (0.00)
54	12	105	0.00 (0.00)	0.27 (0.14)	1.00 (0.19)	0.18 (0.12)
0	12	134	0.08 (0.08)	0.17 (0.11)	1.08 (0.08)	0.17 (0.11)
24/120	9	134	0.44 (0.18)	0.67 (0.17)	1.44 (0.18)	0.33 (0.17)
54/266	12	134	0.25 (0.13)	0.83 (0.11)	0.83 (0.21)	0.42 (0.15)

Table 13. Lesions observed in the liver and pancreas of chinook salmon parr that were exposed to 24 or 54 µg Cr/L to Day 105. The concentrations of chromium were increased from 24 to 120 µg Cr/L and from 54 to 266 µg Cr/L for the remainder of the experiment that ended on Day 134. SEM are in parentheses. The means of lesions scored as none (0), mild (1), moderate (2), or severe (3) are presented. Refer to Appendix 1 and 2 for a glossary of terms and guidelines for scoring sections.

Nominal Chromium (µg/L)	N	Day	Liver		Pancreas
			Glycogen depletion	Hepatocellular lipidosis	Zymogen granule depletion
0	12	105	1.08 (0.26)	0.00 (0.00)	1.00 (0.00)
24	9	105	1.56 (0.18)	0.22 (0.15)	0.67 (0.17)
54	12	105	1.92 (0.19)	0.25 (0.13)	0.58 (0.15)
0	12	134	2.67 (0.14)	0.00 (0.00)	0.58 (0.15)
24/120	9	134	1.67 (0.29)	0.11 (0.11)	0.78 (0.15)
54/266	12	134	2.00 (0.17)	0.00 (0.00)	0.67 (0.14)

Table 14. Measurements of DNA alterations in blood of chinook salmon parr that were exposed to 24 or 54 µg Cr/L to Day 105. The concentrations of chromium were increased from 24 to 120 µg Cr/L and from 54 to 266 µg Cr/L for the remainder of the experiment that ended on Day 134. Different letter designations indicate a significant difference at $P \leq 0.05$, within a sampling day. The difference between the sample CV and the external or internal control is defined as the CV difference and was the discriminating statistic (Misra and Easton 1999).

Nominal Chromium (µg/L)	Day	N	CV DIF
0	105	12	1.730[a]
24	105	9	1.218[b]
54	105	11	1.130[c]
0	134	11	0.411[a]
24/120	134	9	0.450[b]
54/266	134	12	0.506[c]

Note: All probabilities are ≤0.0078

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Appendix 1. Glossary of terms for histology.

Apoptosis – Apoptosis can be a normal process (programmed cell death) associated with development, but increased apoptosis can result from toxicant exposure.

Eosinophilic – Something that stains pink-red-orange with HE stain (e.g. cytoplasm of most cells).

Erythrocytes – Red blood cells

Hematopoiesis - The process of forming red and white blood cells. Hematopoiesis is common in the spleen and in the interstitial tissues that separate kidney tubules.

Hematozylin and Eosin (HE) – Hematoxylin, a blue stain, highlights nucleic acids (e.g. DNA and RNA) that are prominent in the nuclei of cells. Eosin, a pink-red-orange stain, highlights proteins that re common in the cell cytoplasm and in the blood.

Hepatic – Pertaining to the liver.

Hepatocyte – Liver cell.

Hyperplasia – Increase in cell number.

Hypertrophy – Increase in cell size.

Lesion – Any abnormality in tissue, or loss of function of a body part.

Necrosis – The sum of the structural (morphological) changes indicative of cell death and caused by the progressive degradative action of enzymes; it may affect groups of cells or part of a structure or an organ.

Renal – Pertaining to the kidney.

Zymogen granules – Brightly staining (red-orange) spherical structures in the pancreas that store digestive enzymes before they are released into the intestine.

Appendix 2. Guidelines used to score histology.

Kidney

Hematopoietic cells (relative area/volume) – Hematopoiesis is a normal function of the renal interstitium, but in some cases the number of hematopoietic cells was decreased or increased.

Score = 0		area of hematopoietic cells <2/3x area of tubules
Score = 1	mild	area of hematopoietic cells ≥2/3x area of tubules but < 1 1/3x area of tubules
Score = 2	moderate	area of hematopoietic cells ≥ 1 1/3x area of tubules but <2x area of tubules
Score = 3	severe	increased amounts of hematopoiesis, with bands of hematopoietic cells sometimes greater than 100 μm thick

Necrosis of cells lining tubules (Renal Tubular Necrosis)

Score = 0		vessels had no renal tubular necrosis
Score = 1	mild	renal tubular necrosis present, but <4 tubules per cross section
Score = 2	moderate	≥4 or <10 foci of renal tubular necrosis per cross section
Score = 3	severe	≥10 foci of renal tubular necrosis per cross section

Appendix 2. Guidelines used to score histology (continued).

Kidney

Fibrosis around tubules (Peritubular Fibrosis); Affected tubules were surrounded by irregular bands of fibrosis composed of plump fibroblasts and immature collagen.

Score = 0		sections had no peritubular fibrosis
Score = 1	mild	<4 foci of peritubular fibrosis
Score = 2	moderate	≥4 or <10 foci of peritubular fibrosis per cross section
Score = 3	severe	≥10 foci of peritubular fibrosis per cross section

Tubular Dilation

Score = 0		tubules were not dilated
Score = 1	mild	<50% of tubules were dilated
Score = 2	moderate	>50% of tubules were dilated
Score = 3	severe	at least one tubule dilated more than 500 μm in diameter

Appendix 2. Guidelines used to score histology (continued).

Gill

Hypertrophy

Score = 0		lamellar epithelium uniformly simple and squamous
Score = 1	mild	epithelial hypertrophy present but did not involve entire lamellar surface, or thickening is less than the thickness of pillar cells in the section
Score = 2	moderate	epithelial thickening is greater than the thickness of pillar cells in section
Score = 3	severe	foci of thickened lamellae extended at least 500 μm along ≥10 filaments

Apoptosis (death) of chloride cells

Score = 0		no apoptotic chloride cells
Score = 1	mild	apoptotic cells present but < 1 per 400x field
Score = 2	moderate	>1 per 400x field
Score = 3	severe	none were severe

Appendix 2. Guidelines used to score histology (continued).

Spleen

Congestion of blood vessels – Some congestion is normal in the spleen, and lack of congestion may be an indicator of acute stress.

Score = 0		vessels were not congested, and total vascular sectional area was 5% of the spleen volume
Score = 1	mild	total vascular sectional area was >5% but ≤10% of spleen volume
Score = 2	moderate	total vascular sectional area was >10% but ≤25% of spleen volume
Score = 3	severe	total vascular sectional area was >25% of spleen volume

Eosinophilic spherules – Indication of probable erythrocyte damage and/or decreased erythrocyte life span.

Score = 0		no brightly eosinophilic spherules present in section
Score = 1	mild	>1 brightly eosinophilic spherules present in the section, but <5 per 400x field
Score = 2	moderate	>5 but <50 brightly eosinophilic spherules per 400x field
Score = 3	severe	>50 brightly eosinophilic spherules per 400x field

Appendix 2. Guidelines used to score histology (continued).

Liver

Glycogen Depletion – A lesion in hepatocytes. Hepatocytes normally have abundant cytoplasmic glycogen stores characterized by a large volume of clear, irregular, poorly demarcated vacuoles (= glycogen vacuoles).

Score = 0		hepatocytes had abundant glycogen vacuoles
Score = 1	mild	glycogen vacuoles were smaller, but still larger than nuclei
Score = 2	moderate	glycogen vacuoles were smaller than or about equal to nuclear diameter
Score = 3	severe	glycogen vacuoles were absent from most hepatocytes

Lipidosis – A lesion in hepatocytes; lipid appears as clear, round, well-demarcated, cytoplasmic vacuoles (= lipid vacuoles).

Score = 0		hepatocytes had no lipid vacuoles
Score = 1	mild	< 33% of the hepatocytes in the section had lipid vacuoles
Score = 2	moderate	34 – 66% of hepatocytes in the section had lipid vacuoles
Score = 3	severe	> 66% of hepatocytes in the section had lipid vacuoles, or volume of normal lipid vacuoles was greater than nuclear volume

Appendix 2. Guidelines used to score histology (continued).

Pancreas

Zymogen Granule Depletion – During active feeding, exocrine pancreatic cells in healthy fish have abundant brightly eosinophilic cytoplasmic zymogen granules.

Score = 0		abundant zymogen granules
Score = 1	mild	volume of zymogen granules in exocrine cells was >2x nuclear size
Score = 2	moderate	exocrine cells contained zymogen granules, but they were $\leq$2x nuclear size
Score = 3	severe	no distinct zymogen granules in most exocrine cells

Appendix 3. Mean weights and lengths of chinook salmon that were sub-sampled for histology at median hatch. Chinook salmon were exposed to 0, 5, 11, 24, 54 and 120 µg Cr/L. Different letter designations indicate a significant difference at $P \leq 0.05$. SEM in parentheses.

Nominal Chromium (µg/L)	Days	N	Weight (g)	Length (mm)
0	32	4	0.171[a] (0.007)	21.3[a] (0.2)
5	32	4	0.159[a] (0.007)	21.0[a] (0.2)
11	32	4	0.150[b] (0.007)	20.7[a] (0.2)
24	32	4	0.165[a] (0.007)	21.2[a] (0.2)
54	32	4	0.156[a] (0.007)	20.8[a] (0.2)
120	32	4	0.163[a] (0.007)	20.8[a] (0.2)

Appendix 4. Mean weights and lengths of chinook salmon that were sub-sampled for physiology during the alevin stage (midway between hatch and swim-up). Chinook salmon were exposed to 0, 5, 11, 24, 54 and 120 µg Cr/L. Different letter designations indicate a significant difference at $P \leq 0.05$. SEM in parentheses.

Nominal Chromium (µg/L)	Days	N	Weight (g)	Length (mm)
0	70	4	0.221[a] (0.005)	31.2[a] (0.2)
5	70	4	0.220[a] (0.005)	31.2[a] (0.2)
11	70	4	0.219[a] (0.005)	31.2[a] (0.2)
24	70	4	0.218[a] (0.005)	31.1[a] (0.2)
54	70	4	0.223[a] (0.005)	31.6[a] (0.2)
120	70	4	0.228[a] (0.005)	31.6[a] (0.2)

Appendix 5. Mean weights and lengths of chinook salmon that were sub-sampled for physiology and histology at the median swim-up date and the termination of chromium exposure. Chinook salmon were exposed to 0, 5, 11, 24, 54 and 120 µg Cr/L. Different letter designations indicate a significant difference at $P \leq 0.05$. SEM in parentheses.

Nominal Chromium (µg/L)	Days	N	Weight (g)	Length (mm)
0	83	4	0.232[a] (0.004)	33.5[a] (0.2)
5	83	4	0.240[a] (0.005)	33.4[a] (0.2)
11	83	4	0.228[a] (0.005)	32.0[b] (0.2)
24	83	4	0.245[a] (0.005)	32.1[b] (0.2)
54	83	4	0.227[a] (0.006)	31.8[b] (0.2)
120	83	4	0.234[a] (0.005)	33.2[a] (0.2)

Appendix 6. DNA strand breakage in chinook salmon parr as measured by gel electrophoresis. Mean concentrations presented as the log of kilobase pairs in gill and liver tissues collected during an experiment where chinook salmon were exposed to 24 or 54 µg Cr/L to Day 105. The concentrations of chromium were increased from 24 to 120 µg Cr/L and from 54 to 266 µg Cr/L for the remainder of the experiment that ended on Day 134. Different letter designations indicate a significant difference at $P \leq 0.05$ within a tissue and within a sample day. SEM in parentheses.

Nominal Chromium (µg/L)	Day	N	Gill	Liver
0	105	4	4.2[a] (1.1)	3.4[a] (0.4)
24	105	3	3.1[a] (0.29)	3.1[a] (0.7)
54	105	4	12.6[a] (6.88)	4.9[a] (0.9)
0	134	4	4.1[a] (1.5)	3.3[a] (0.4)
24/120	134	3	3.3[a] (0.5) N=2	2.8[a] (0.3)
54/266	134	4	3.8[a] (1.0)	3.0[a] (0.4)